Ethambutol partitioning in tuberculous pulmonary lesions explains its clinical efficacy

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Clinical trials and practice have shown that ethambutol is an important component of the first line tuberculosis (TB) regime. This contrasts the drug's rather modest potency and lack of activity against non-growing persisting mycobacteria. The standard plasma-based pharmacokinetic-pharmacodynamic profile of ethambutol suggests that the drug may be of limited clinical value. Here we hypothesized that this apparent contradiction may be explained by favorable penetration of the drug into TB lesions. First we utilized novel in vitro lesion pharmacokinetic assays and predicted good penetration of the drug into lesions. We then employed mass spectrometry imaging and laser capture microdissection coupled to liquid chromatography and tandem mass spectrometry (LCM/LCMS) to show that ethambutol indeed accumulates in diseased tissues and penetrates the major human-like lesion types represented in the rabbit model of TB disease with a lesion-to-plasma exposure ratio ranging from 9 to 12. In addition, ethambutol exhibits slow but sustained passive diffusion into caseum to reach concentrations markedly higher than measured in plasma at steady state. The results explain why ethambutol has retained its place in the first line regimen, validate our in vitro lesion penetration assays and demonstrate the critical importance of effective lesion penetration for anti-TB drugs. Our findings suggest that in vitro and in vivo lesion penetration evaluation should be included in TB drug discovery programs. Finally, this is the first time that LCM/LCMS is used to quantify a small molecule at high spatial resolution in infected tissues, a method that can easily be extended to other infectious diseases.
In 2015, tuberculosis (TB) became the single leading infectious cause of death in adults, surpassing HIV (1).

Ethambutol (EMB) is part of the four-drug regimen used to treat drug susceptible TB in the initial two-month intensive phase of therapy. It was introduced in the 1980's, based on a series of large clinical trials by the British Medical Research Council (2, 3). These trials indicated that EMB lacked sterilizing activity but was useful in protecting against the emergence of resistance to the three other drugs: isoniazid, rifampicin and pyrazinamide (3, 4).

As a cell wall synthesis inhibitor, EMB is bactericidal against replicating bacilli but has limited potency against slow-growing and non-replicating bacteria (5), in line with its early bactericidal activity (second best after INH (6)) and reported lack of sterilizing activity. In vitro, the activity of EMB is modest but similar against extracellular bacteria in liquid culture and intracellular bacilli in monocytes (7) or THP-1 macrophages (8). In mouse models, EMB is mostly static, resulting in a 2-log difference in bacterial burden between untreated and treated animals after 28 days of daily therapy at the human equivalent dose ((9) and our unpublished data). Globally, the pharmacokinetics of EMB are less subject to inter-individual variability than other first line agents (10, 11).

Absorption is not impacted by food (12), the drug exhibits low plasma protein binding and very good distribution into uninfected mouse tissues (13). There are anecdotal reports of 8 to 10 times higher levels in the lungs of patients who underwent pulmonary surgery for conditions other than TB (14). Favorable distribution was also demonstrated in the lung tissue and cells of non-infected primates (15). In TB patients, EMB levels were similar in pleural fluid and plasma (16), and more than 20 times higher in peripheral blood mononuclear cells than in plasma (17). However, nothing is known about the distribution, absolute concentrations and spatial partitioning of EMB in pulmonary TB lesions.

Despite its modest activity in vitro and in mouse models, EMB found its place in the first ‘short-course chemotherapy’ because this regimen delivered the best clinical outcome among many other combinations...
In a pivotal trial where either streptomycin or EMB was added to isoniazid, rifampicin and pyrazinamide, the proportion of patients who converted their sputum culture by 2 months was identical in both arms (3, 18), thus conferring a clear advantage to EMB, an oral drug with a less toxic profile than streptomycin. In addition, EMB is retained in the regimens of multidrug resistant TB patients (resistant to isoniazid and rifampicin) who are sensitive to EMB. Some non-tuberculous mycobacterial infections, a rapidly emerging class of lung diseases, are also treated with EMB as part of multidrug regimens (19, 20). The present study was undertaken to investigate the distribution of EMB in the lung tissue and lesions of rabbits with active TB disease. Does EMB reach the various lesion types and compartments at concentrations that are sufficient to exert bactericidal and intracellular activity? Our findings provide a rational explanation for the contribution of EMB to first line TB treatment despite its modest potency and plasma pharmacokinetics.
Macrophage uptake assays

EMB uptake into THP-1 macrophages was performed as previously described (21). Murine bone marrow derived macrophages were subjected to classical (M1) or alternate (M2) activation. M1 macrophages are microbicidal and mediate resistance to intracellular pathogens, while M2 macrophages support anti-inflammatory functions such as tissue repair and tumor progression. Bone marrow was harvested from C57Bl/6 mice and plated in D-10 media (DMEM with 10%FBS) supplemented with 20% L-cell conditioned media (L929 cells secrete Macrophage Colony-stimulating factor which differentiates bone marrow cells into macrophages). After 7 days, the cells (now macrophages) were harvested using a cell stripper and replated in D-10 with 2% L-cell. For M1 polarization, cells were treated with 100 U/ml of γ-IFN overnight. For M2 polarization, cells were treated with IL-4 (20 ng/ml) and IL-13 (10 ng/ml) for two days. The drug penetration assay was conducted in a 24-well plate format with $1 \times 10^6$ cells per well. EMB was added at a final concentration of 5µM for 30min. Cells were then washed and lysed with distilled water prior to EMB quantitation by liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS). Briefly, 100 µL of cell lysate was sampled from each well, combined with 35 µL of untreated cell lysate spiked with 1 µg/mL diclofenac as internal standard, and 15 µL of 1:1 MeOH:H₂O. Each sample was vortexed for 5 minutes, centrifuged for 5 minutes at 10,000 rpm, and 100 µL of supernatant was transferred to deep 96-well plate for LC/MS-MS analysis. To quantify the total number of cells/well, 50 µL of each cell lysate was removed from each well and added to a clear bottom black-sided 96-well plate. 50 µL of deionized water and 100 µL of PicoGreen (Life Technologies) were added, and the plates were incubated for 2–5 min, protected from light. Fluorescence was measured at 520 nm (excitation wavelength 480 nm). Samples were blank subtracted, and cell number interpolations were made from a standard curve.
Human bone marrow derived monocytes were isolated from the blood of three independent donors by ficoll separation followed by purification with CD14+ MicroBeads (StemCellTechnologies catalog #18058). Approximately 2.10^5 monocytes were plated in 500 µL of media (RPMI supplemented with 5% FBS; 1% penicillin/streptomycin; 1% HEPES; 10% Human serum; 0.01% M-CSF) in 24-well plates and incubated at 37°C with 5% CO₂ for 2 days, after which half of the culture medium was replaced with fresh medium. After 4 more days of incubation, the culture medium was replaced with either fresh medium containing a 1:5000 dilution of heat-inactivated M. tuberculosis (BEI Resources cat. NR-14819) or fresh medium in the control wells, followed by 24h incubation at 37°C with 5% CO₂ (The stock of heat inactivated M. tuberculosis was vortexed with 3mm diameter glass beads to disrupt the bacterial aggregates; the remaining large aggregates were let to sediment for 30-40 minutes and the supernatant was frozen at -80°C in aliquots). Next, the medium was replaced with medium containing 40 µM EMB, and the plates were incubated for 30 minutes. Each well was washed twice with ice cold PBS, the cells were lysed with 250 µL ddH₂O and incubated at 37°C for 1h. Prior to drug quantitation, 50 µL were removed and used to quantify the viable macrophages using the PicoGreen assay as described above.

Caseum binding assay

The caseum binding assay was carried out by rapid equilibrium dialysis as previously described (22). Briefly, caseum was diluted 10-fold in PBS, homogenized, and spiked to give the final incubation concentration of 5 µM. The spiked matrix was placed in the sample chambers and the buffer chambers were filled with 350 µl of PBS. The plates were then covered with adhesive seals and incubated at 37°C for 4h on an orbital shaker set at 300 rpm. Following incubation, samples were removed from both chambers and processed by adding an organic solvent mixture (1:1 methanol/acetonitrile) prior to LC-MS quantification. Fraction unbound (fu) in plasma and diluted caseum was calculated as the ratio between free (buffer chamber) and total drug (sample chamber) concentrations, as shown in equation (1). A dilution factor of 10 (D=10) was applied to the calculation of fu in...
Rabbit infection and drug administration

All animal studies were performed in Biosafety Level 3 facilities and approved by the Institutional Animal Care and Use Committee of the New Jersey Medical School, Rutgers University, Newark, NJ. Female New Zealand White (NZW) rabbits (Millbrook Farm, Concord, MA), weighing 2.2 to 2.6 kg, were maintained under specific pathogen-free conditions and fed water and chow ad libitum. The rabbits were infected with M. tuberculosis HN878, using a nose-only aerosol exposure system as described (25). Three hours post-infection, one rabbit from each round of infection was sacrificed to determine the bacterial load implanted in the lungs. At defined time points from 16 to 21 weeks post-infection, 10 rabbits received a single dose of 100 mg/kg EMB formulated in 40% sucrose by oral gavage, and 3 rabbits received 7 daily doses of 100 mg/kg EMB to reach steady state. These time points were selected to ensure that both cellular and necrotic granulomas had formed and reached a size sufficient to allow dissection of individual lesions. At 16 to 21 weeks post infection, all rabbits have both cellular and necrotic granulomas. Cavities are occasional and were treated as necrotic lesions in this study (i.e. the caseum was not separated from the cavity wall for LC/MS-MS analysis). Blood was collected from the central ear artery of each rabbit pre-dose, and at several time points between drug administration and necropsy (typically 0.5, 1, 2, 4, 6, and/or 24h following drug administration). Groups of 2 to 4 rabbits were euthanized at 2...
(4 rabbits), 6 (4 rabbits) and 24h (2 rabbits) post-dose. These time points were selected based on the plasma PK profile to capture the $C_{\text{max}}/T_{\text{max}}$ (2h), the end of the distribution phase (6h) and the trough or $C_{\text{min}}$ (24h). All blood samples were centrifuged at 4,000rpm for 5min and the supernatants (plasma) were transferred and stored at -80°C until analyzed by high-pressure liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS).

**Lesion dissection and processing**

The right and left lungs were removed and weighed for analytical drug measurement and histopathology. From each lung lobe, individual granulomas and uninvolved (non-diseased) lung tissue sections were dissected, sized, weighed and recorded. Lesions weighing less than 5mg were pooled. Special care was taken to remove the uninvolved lung tissue surrounding each granuloma. The samples collected from each rabbit were classified as uninvolved lung, necrotic or cellular granulomas, cavity wall or cavity caseum. When necrotic granulomas were greater than 7mm, they were dissected so that the lesion wall and the caseous material within could be stored and analyzed separately. Lesions collected for laser-capture microdissection were left embedded in the surrounding tissue, and snap-frozen in liquid nitrogen vapor as described previously (26). All samples were stored in individual 2ml tubes at -80°C.

Prior to drug quantitation by LC/MS-MS, all tissue samples were homogenized in approximately, but accurately recorded, 4 volumes of phosphate buffered saline (PBS). Homogenization of all tissue samples was achieved using a FastPrep-24 instrument (MP Biomedicals) and 1.4mm zirconium oxide beads (Precellys). Extraction was performed by adding 180µl of 1:1 acetonitrile/methanol containing 100ng/mL stable labeled EMB-d10 to 20µl of plasma or homogenized tissue sample and 20µl of 1:1 acetonitrile/water (ACN:H$_2$O). The mixtures were vortexed for 5min and centrifuged at 4,000rpm for 5min, separating the precipitated proteins from the extracts that were then transferred for LC/MS-MS analysis.

**MALDI mass spectrometry imaging of EMB in rabbit lesions**
Twelve \(\mu\)m thick tissue sections were cut from gamma-irradiated rabbit lung biopsies using a Microm HN505 N (Walldorf, Germany) and thaw-mounted onto stainless steel slides (MALDI-MSI) or standard glass microscope slides (for H&E staining).

Plates containing tissue sections for MALDI-MSI were allowed to reach room temperature for 15 minutes prior to opening of the containers. 2,5-Dihydroxybenzoic acid matrix (25mg/mL in 60% Methanol/0.1% TFA) (Sigma-Aldrich, St Louis, MO) was applied to the tissues via the TM-Sprayer automated MALDI tissue prep device (HTM Technologies, North Carolina) using the following optimized conditions: 0.04mL/min flow rate; 60°C nozzle temperature; 1.3mm/second raster speed; 25 passes over the tissue. EMB-d10 (C/D/N Isotopes, Quebec) was added to the matrix at 5pmol/\(\mu\)L as an internal standard.

MALDI-MSI acquisition was performed MALDI LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a resolution of 60,000 at m/z 400, full width at half maximum. Imaging data was acquired in full scan mode to maximize sensitivity and drug peak identities were confirmed by acquiring several MS/MS spectra directly from the dosed tissues.

Instrument parameters were tuned and optimized on spiked EMB drug standard on stainless steel plates and control mouse lung tissue. Limit of detection (LOD) for MALDI-MSI analysis of EMB was 1\(\mu\)g/g calculated as described previously (21).

Spectra were acquired in positive mode across the mass range m/z 190-400. A laser energy of 20\(\mu\)J was applied and 5 laser shots were fired at each position (total of 1 microscan per position). The laser step size was set at 50\(\mu\)m which enabled small necrotic areas within lesions to be resolved without overlapping of the laser spot on adjacent acquisitions. Thermo ImageQuest software (\(v1.01\)) was used to reconstruct 2D ion images. Normalized ion images of EMB were generated by dividing EMB [M+H]\(^+\) signal (m/z 205.193 \(\pm\) 0.003) by EMB-d10 [M+H]\(^+\) signal (m/z 215.039 \(\pm\) 0.003).

Laser-capture microdissection of rabbit tissue slices
Twenty five µm thick tissue sections were cut from gamma-irradiated rabbit lung biopsies using a Microm HN505 N (Walldorf, Germany) and thaw-mounted onto 1.4 µm thick Leica PET-Membrane FrameSlides (Herborn, Germany) for laser capture microdissection. Tissue sections were immediately stored in sealed containers at -80°C. Adjacent 12 µm thick tissue sections were thaw-mounted onto standard glass microscopy slides for H&E and Ziehl-Neelsen staining.

Cellular, necrotic (caseum), and uninvolved lung lesion areas totaling 5 million µm² were dissected from between 3 and 6 serial lung biopsy tissue sections using a Leica LMD6500 system (Buffalo Grove, IL). Areas of cellular and caseous lesion were identified optically from the brightfield image scan and by comparison to the adjacently-sectioned H&E reference tissue. Pooled dissected lesion tissues were collected into 0.25 mL standard PCR tubes and immediately transferred to the -80°C. Prior to analysis, the tubes were thawed at room temperature for 30 minutes. 50 µL of extraction solution (ACN/MeOH (1/1) with 10 ng/mL Verapamil and 100 ng/mL EMB-d10) was added to each tube, which were then sonicated for 5 minutes and centrifuged at 10,000 RPM for 5 minutes at room temperature. 40 µL of supernatant was transferred for LC/MS-MS analysis and diluted with an additional 40 µL of MilliQ water.

Neat 1 mg/mL DMSO stocks for all compounds were diluted serially in 50/50 Acetonitrile water to create standard curves and quality control spiking solutions. 2 µL of neat spiking solutions were added to 2 µL of lesion homogenate and extraction was performed by adding 50 µL of extraction solution (ACN/MeOH (1/1) with 10 ng/mL Verapamil and 100 ng/mL EMB-d10). Extracts were vortexed for 5 minutes and centrifuged at 10,000 RPM for 5 minutes. 40 µL of supernatant was transferred for LC/MS-MS analysis and diluted with an additional 40 µL of MilliQ water. Previously optimized LC/MS-MS parameters were used for analysis (see LC/MS-MS section). The total tissue volume of each pooled sample was determined based on the surface area of the pooled sections and the 25µm tissue thickness. A dilution factor was used to normalize the tissue volumes with the standard curve for quantification. Note that this analysis was run on three year-old tissue samples; despite
having been stored at -80°C, EMB had partially degraded which explains the 2 to 3 fold lower concentrations than measure by standard LC/MS-MS using lesion homogenates.

**HPLC-mass spectrometry method**

Neat 1 mg/mL DMSO stocks for all compounds were serially diluted in 50/50 acetonitrile/water to create standard curves and quality control spiking solutions. Twenty microliters of neat spiking solutions were added to 20 µL of drug free plasma or control tissue homogenate, and extraction was performed as above. NZW control plasma treated with K$_2$EDTA was obtained from Bioreclamation and used to build standard curves. Gamma-irradiated lung, lesion, and caseum samples from tuberculosis infected NZW rabbits were used as control tissues and homogenized as above. LC/MS-MS analysis was performed on a Sciex Applied Biosystems Qtrap 4000 triple-quadrupole mass spectrometer coupled to an Agilent 1260 HPLC system to quantify EMB in the plasma and lung samples. Chromatography was performed with an Agilent Zorbax SB-C8 column (4.6x50 mm; particle size, 3.5 µm) using a reverse phase gradient elution with ion pairing reagents. The aqueous mobile phase consisted of 6mM heptafluorobutyric acid (HFBA) and 10 mM ammonium hydroxide (NH$_4$OH) in water and the organic mobile phase consisted of 3 mM HFBA and 10 mM NH$_4$OH in 95:5 ACN:H$_2$O. Multiple-reaction monitoring (MRM) of parent/daughter transitions in electrospray positive-ionization mode was used to quantify the analytes. The MRM transitions were EMB (205.1/116.2) and EMB-d10 (215.10/123.20). Sample analysis was accepted if the concentrations of the quality control and standards were within 20% of the nominal concentration. Data processing was performed using Analyst software (version 1.6.2; Applied Biosystems Sciex).

EMB and the EMB-d10 labeled internal standard were purchased from Alfa Aesar and C/D/N isotopes, respectively.

**Pharmacokinetic modeling**

Concentration-time data for EMB in plasma, lesion homogenates and uninvolved lung homogenates from each rabbit – determined by LC/MS-MS – were modeled using a population methodology. Data were fit to nonlinear...
mixed-effects models with first-order conditional estimation method as implemented in the software NONMEM (version 7.3; ICON Development Solutions, Ellicott City, MD). Graphical, statistical, and exploratory analyses were conducted using the open-source software, R (version 3.3.1). The Xpose (version 4.0) package, implemented within R, was used for graphical evaluations and visual predictive checks. A structural PK model was fit to the plasma PK data. Second, a full model describing penetration into uninvolved lung, caseous and cellular lesions was fit using an ‘effect compartment’ approach as described by Savic \textit{et al.} (27, 28). Additive and proportional error models of residual variability were explored for plasma, lung, and lesions. Finally, all parameters were re-estimated simultaneously using all available data. The statistical significance of parameter addition was judged on the basis of a log-likelihood ratio test, based on reduction of the objective function value (OFV) with an acceptance \( P \) value of 0.05. The final PK model that optimally described the data was a two-compartment structural model with first-order absorption and elimination according to the following ordinary differential equations:

\[
\frac{dC_a}{dt} = -k_a \times C_a \quad (1)
\]

\[
\frac{dC_c}{dt} = k_a \times C_a - (k_{cp} + Cl/V_c) \times C_c + (k_{pc} \times C_p) \quad (2)
\]

\[
\frac{dC_p}{dt} = k_{cp} \times C_c - k_{pc} \times C_p \quad (3)
\]

Equations 1-3 represent a two-compartment plasma PK model with first-order absorption and elimination, where \( C \) represents the amount of EMB (in mg) in the absorption (a), central (c) and peripheral (p) compartments, \( V_c \) and Cl represent central compartment volume and EMB clearance, and \( K_{cp} \) and the \( K_{pc} \) are inter-compartment rate constants, respectively.

\[
\frac{dC_l}{dt} = k_{cl} \times \left( R_{cl} \times \frac{A_l}{V_c} - C_l \right) \quad (4)
\]
Equations 4-6 represent EMB penetration within lung, caseous and cellular lesions, where \( C \) represents the amount of EMB (in mg) in the uninvolved lung (l), cellular (cell) and caseous (cas) lesions. \( k_{cel} \), \( k_{cell} \) and \( k_{cas} \) are inter-compartment rate constants, \( R_{cl}, R_{ccl} \) and \( R_{ccas} \) represent penetration coefficients between the central compartment and lung or lesion, and \( A_c/V_c \) is the drug concentration in plasma at time \( t \).

Model validation. The final models of EMB were validated using a nonparametric bootstrap resampling technique. A total of 1000 bootstrap datasets were generated based on random sampling with replacement. The final model was fit to the bootstrap data and PK parameters re-estimated. Measures of central tendency and dispersion and the 95% confidence interval (CI) for each parameter value were calculated and compared with the original parameter value’s estimates.

Simulations and exploration of humanized-regimens. Predicted AUC\(_{0-24}\) and \( C_{\text{max}} \) in plasma, lung and lesions were computed. Visual predictive checks were performed to evaluate the simulation properties of the final model and to explore tissue kinetics following administration of doses predicted to achieve human-like systemic exposures based on published data (10, 12, 29).

Pharmacokinetic-pharmacodynamic exposure target selection. PK-PD target indices were identified for EMB from available literature. The drug exposure index that best explains microbial kill of \( M. \) tuberculosis by EMB is the ratio of the AUC\(_{0-24}\) to the MIC (AUC\(_{0-24}\)/MIC). An AUC\(_{0-24}\)/MIC ratio of 119 has been associated with 90% maximal effective concentrations (EC\(_{90}\)) for EMB (30). Target attainment analysis was performed based on the
distribution of wild-type MICs for EMB obtained from published epidemiological data based on clinical isolates tested according to standardized EUCAST methodology (31, 32).
RESULTS

In vitro lesion pharmacokinetics

Previous work by our group indicated that drug distribution and partitioning within lesions is a function of uptake into macrophages and binding to caseum macromolecules (21, 22). To confirm these findings and predict the behavior of EMB at the interface between the cellular rim and necrotic core of TB lesions, we measured EMB caseum binding and uptake into macrophages of different origins. The average caseum free fraction was high at 35-38% (SD 4-5%), predicting favorable passive diffusion through non-vascularized caseum. EMB uptake was quantified in THP-1 macrophages, classically activated (M1), alternatively activated (M2) and non-activated (M0) murine bone marrow derived macrophages (MDM), and human MDM isolated from three independent donors followed by activation with heat-killed *M. tuberculosis*. EMB reached higher intracellular than extracellular concentrations in all cell types tested. The average intracellular to extracellular concentration (I/E) ratio after 30 minutes of drug exposure ranged between 5 and 20, with THP-1 cells showing the lowest intracellular uptake. Overall, uptake was not significantly affected by activation status (Table 1). This favorable cellular uptake predicted good penetration into cellular lesions.

EMB penetration in cellular and necrotic lesions

To quantify the distribution of EMB in pulmonary lesions in vivo, a cohort of 10 New Zealand White rabbits was infected with *M. tuberculosis* HN878 until they developed mature lesions (*Figure S1*), at which point they received a single oral dose of EMB 100mg/kg either 2h, 6h or 24h prior to lung and lesion dissection. EMB concentrations were measured in plasma collected serially from pre-dose to necropsy, and in dissected lung tissue and whole lesions sorted as either cellular or necrotic granulomas (*Table S1*). Concentration-time data for EMB in plasma, lesion homogenates and uninvolved lung were modeled using a population methodology. The final PK model that optimally described the data was a two-compartment structural model with first-order absorption and elimination. The parameter estimates and associated estimation of errors are summarized in...
Table 2. Goodness-of-fit plots are shown in Figure S2. Significant tissue partitioning of EMB was observed; the mean steady state AUC$_{0-24}$ measured in uninvolved lung, cellular lesions and caseous lesions were 11.8, 12.2 and 8.8-fold higher than the plasma AUC$_{0-24}$, respectively (Figure 1A-D). Distribution into necrotic lesions was slightly slower than in lung and cellular lesions, likely due to slower diffusion into avascular caseum than in well vascularized cellular layers. Overall, EMB accumulated rapidly in lung tissue and lesions at markedly higher concentrations than measured in plasma.

Spatial quantitation of EMB in lesion compartments

To visualize the relative partitioning of EMB at the interface between cellular and necrotic regions of caseous granulomas, we acquired MALDI mass spectrometry images of EMB in necrotic granulomas and cavities obtained from rabbits that had received either a single or multiple EMB doses (100 mg/kg). Although the poor ionization of EMB provided limited sensitivity, the ion maps clearly showed good distribution in the fully cellular granuloma, and higher accumulation in the cellular layers than in the necrotic foci of closed caseous nodules and cavities (Figure 2). To quantify the relative partitioning of EMB into cellular and necrotic lesion compartments, we used laser-capture micro-dissection (LCM) coupled to LC/MS-MS (Figure S3) and measured EMB concentration in micro-dissected caseum, cellular rim, and uninvolved lung from the lesions of rabbits that had received 7 daily doses of EMB. Lesion samples were collected 6h after the last EMB dose, or at the end of the distribution phase. This experiment was carried out at steady state since we have found that passive drug diffusion into caseum can be a slow process (21). To validate the LCM methodology, EMB was also quantified in whole dissected lesions from the same rabbits by LC/MS-MS (Figure 3A). The results confirmed the striking accumulation of EMB in uninvolved lung and cellular rims relative to plasma. EMB levels measured in caseum were lower than in the adjacent cellular layers but still markedly higher than in plasma (Figure 3A, empty bars). EMB concentrations measured in lung tissue and whole cellular lesions were in very good agreement with the LCM dataset (Figure 3A, solid bars, and Table S2). The caseum-to-cellular concentration ratio was slightly higher.
after 7 daily EMB doses than following a single dose (Figure 3B), suggesting that EMB diffuses slowly into caseum and accumulates in this compartment upon multiple dosing.

Pharmacokinetic-pharmacodynamic target attainment at therapeutic doses

Next we applied the model of EMB lesion penetration to determine whether adequate lesion concentrations are achieved in the lung lesions of patients receiving a standard dose. Assuming dose proportional PK, a simulated daily dose of 200 mg/kg EMB in rabbits resulted in human-equivalent systemic exposures achieved in adults receiving daily doses of 1200 mg daily (Table 3) (10, 12). Figure 4 shows the relative MIC coverage of EMB in the plasma and lung tissue sub-compartments. At human-equivalent doses, EMB exposure in plasma did not achieve an AUC₀-24/MIC of 119 (29, 30) in the majority of subjects across the MIC range studied. By contrast, exposures within uninvolved lung and both cellular and caseous lesions were associated with significantly higher probability of target attainment; for isolates with an MIC ≤ 2 mg/L, the probability of target attainment in all tissue compartments exceeded 0.9.
EMB has been part of the first line regimen since the 1980’s, yet it is a moderately potent drug with little to no activity against non-replicating bacteria. Clinically achieved concentrations in plasma are above the minimum bacteriostatic or bactericidal concentrations for a relatively small fraction of the dosing interval (11). Nevertheless, large clinical trials where EMB was replaced with either moxifloxacin or gatifloxacin, two fluoroquinolones with superior plasma based PK-PD properties compared to EMB, failed to achieve treatment shortening or lower relapse rates (33, 34). Here we show that extensive and sustained accumulation of EMB into cellular and necrotic lesions likely drives PK-PD target attainment, providing a rational explanation for the contribution of EMB to the first line regimen. In addition to global distribution from plasma into lung lesions, we were particularly interested in the relative distribution of EMB between cellular and necrotic compartment, since we have shown that caseum is often the most problematic niche in terms of drug penetration (21, 26, 35). To this aim, we resorted to laser-capture microdissection coupled to mass spectrometry (LCM/MS) in order to obtain spatial quantitation of EMB at the sub-lesional level. We show that EMB accumulates in cellular layers relative to plasma but that caseum levels do not exceed those measured in plasma, suggesting that the free EMB fraction equilibrates passively between plasma and necrotic/acellular compartments. This is the first time that LCM/MS is used to quantify a small molecule at high spatial resolution in infected tissues. We have previously proposed that drug partitioning at the caseum/cellular interface of necrotic lesions is a function of uptake into macrophages, binding to caseum macromolecules, and cLogP (21, 22). Only drug molecules that are not taken up by macrophages and not bound to macromolecules are free to diffuse passively through non-vascularized caseum. Thus low macrophage uptake and low caseum binding correlate with favourable partitioning into necrotic foci relative to the surrounding cellular rim, and favourable diffusion through caseum inversely correlates with hydrophobicity (cLogP). EMB (cLogP = 0.14) demonstrated high macrophage uptake and low caseum binding, which resulted in slow but substantial diffusion into caseum at steady state. Indeed, the AUC measured in cellular lesions was approximately 10 times higher than in plasma,
in line with the intracellular uptake of EMB in resting and activated primary macrophages of mouse and human origin (Table 1). This result was also consistent with the 20-fold accumulation reported in peripheral blood mononuclear cells (17). Spatial quantitation of EMB in the cellular layers and necrotic core of rabbit lesions revealed progressive diffusion into caseum, whereby the caseum-to-plasma concentration ratio was around 1 after a single dose and increased to 30-40 after 7 daily doses, to reach 5 to 10 μg/g in caseum throughout the dosing interval at steady state. Such distribution pattern is reminiscent of rifampicin (21) which is more highly bound to caseum proteins but exhibits lower uptake into macrophages. These results lend support to the emerging theory that distribution into caseum is a balance between high macrophage uptake limiting caseum penetration and low protein binding allowing free diffusion into caseum (26) (36). Overall, EMB further validates the use of these two in vitro assays to predict intra-lesional partitioning of small molecules.

In TB patients receiving 15 to 20 mg/kg, the EMB peak plasma concentration (C_{max}) and AUC range between 2.3 and 2.8 μg/mL and 15 to 25 μg.h/mL, respectively (10, 12, 29). To predict EMB concentrations in the pulmonary granulomas of TB patients, we modeled the rabbit dataset and simulated plasma and lesion concentrations at the human equivalent dose of 200mg/kg. The activity of EMB against intracellular M. tuberculosis ranges between 1 μg/mL to achieve a static effect (37) and 5 μg/mL to achieve cidality (38, 39). If one applies the modeled distribution of EMB in cellular lesions relative to plasma, these concentrations are expected to be achieved and maintained throughout the entire dosing interval in the lesions of rabbits receiving a humanized dose (Figure S4) and likely in TB patients. Thus, the PK-PD metrics of EMB in cellular lesions as well as in the cellular rim of necrotic lesions points to intracellular bacilli as a major target population in human lung lesions. Since EMB is poorly active against non-replicating M. tuberculosis (5) such as those found in caseum, it is unlikely to sterilize the caseous foci of necrotic lesions despite its favorable distribution in this compartment at steady state. Such prediction is consistent with the lack of sterilizing activity of EMB (3, 4, 18).
EMB is used in the treatment of nontuberculous mycobacterial (NTM) disease, particularly in patients with *M. avium* lung disease (19, 20), the pathology of which is similar to TB (40). Our findings thus extend to mycobacterial diseases in general, a growing public health threat in developed countries (41).

The following assumption and limitation must be noted. Dose proportional exposure was assumed to simulate tissue distribution at the human-equivalent dose of 200 mg/kg for the computation of target attainment. Only a small number of rabbit lesions were subjected to laser-capture micro-dissection and LCMS analysis following a single dose and at steady state. Larger rabbit cohorts will be required to characterize the dynamics of diffusion into caseum over time, and determine the time required for EMB to reach steady state equilibrium between the caseous and cellular lesion compartments. While the rabbit remains a model and does not recapitulate all aspects of human pathology, we have so far observed similar patterns of lesion partitioning in rabbits and humans for rifampicin, moxifloxacin and pyrazinamide (Dartois & Savic, unpublished). In summary, we have used quantitative and imaging methods to characterize the penetration and partitioning of EMB in cellular and necrotic lung lesions. While EMB plasma concentrations appear inadequate to attain proposed PK-PD targets associated with efficacy, extensive tissue partitioning results in significantly higher exposure within pulmonary tissues, which likely drives the clinical efficacy of EMB. Lesion-centric PK-PD analyses thus provide the first step towards a rational explanation for the contribution of EMB to first line TB treatment.

**ACKNOWLEDGEMENTS**

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Pyrazinamide Treatment Responses are Affected by the Heterogeneity in Pulmonary Lesions in Mycobacterium tuberculosis Infected C3HeB/FeJ Mice. ACS Infect Dis 2:251-267.


FIGURE LEGENDS

Figure 1. Visual predictive check (VPC) for EMB concentration versus time, stratified by compartment or tissue type. (A) plasma, (B) lung, (C) cellular lesions, (D) caseous/necrotic lesions. Observed data are shown as open circle symbols. Dashed and solid lines delineate the 5th, 50th, and 95th percentile of observed data. Shaded areas encompass the 95% confidence interval for the equivalent percentiles as predicted by the final model.

Figure 2. Spatial distribution of EMB in cavity (C), cellular granuloma (CG) and necrotic granulomas (NG) by MALDI mass spectrometry imaging (MSI). The left panels shows ion maps of EMB ([M+H]+ m/z 205.193 ± 0.003) in a cavity (top), two necrotic granulomas and a cellular granuloma (bottom). The intensity scale is shown on the left. The middle panel is a hematoxylin/eosin staining of the tissue section directly adjacent to the section used for MALDI MSI. The right panel shows the optical image of the section used for MALDI MSI prior to matrix application and image acquisition.

Figure 3. Spatial quantitation of EMB in lung and lesion compartments. (A) The left half of the panel shows absolute EMB concentrations measured by LC/MS-MS in lung and distinct regions of necrotic granulomas, laser-captured and dissected from thin tissue sections as shown in the upper left corner (see Figure S3 for detailed procedure). (B) The right half of the panel shows data acquired by LC/MS-MS in tissue homogenates collected by standard dissection of uninvolved lung, whole cellular lesions and whole necrotic lesions. Both rabbits 2551 and 2537 received 100 mg/kg EMB daily for 7 days, and lesions were dissected 6h after the last dose (steady state).

The concentrations required to kill 99% of extracellular replicating bacilli (MBC99) and 99% of intracellular bacilli in macrophages (iMBC99) are indicated (5, 38). (B) Comparison of EMB concentration ratios between lung and cellular or necrotic lesion compartments following a single dose and at steady state. Absolute EMB concentrations were measured by LC/MS-MS in uninvolved lung, cellular rim and necrotic core of caseous granulomas, laser-captured and dissected from thin tissue sections as shown in (A).
Figure 4. (A) Steady-state AUC₀-2₄ distribution from 1,000 simulated subjects (rabbits) receiving 200 mg/kg EMB to achieve plasma AUC equivalency to adults receiving 1,200 mg as reported by Denti et al (10). Red, orange, green and blue bars are AUC distributions for plasma, lung, cellular lesions and caseous lesions, respectively. (B) Probability of target attainment for a PK-PD target AUC₀-2₄/MIC of 119 to achieve 90% of maximal kill (30) in 1000 simulated subjects receiving a daily dose of 200 mg/kg. The right Y axis and corresponding open bars show MIC distributions for *M. tuberculosis* as reported by Schön et al. (31) and Kaniga et al. (32). Red, orange, green and blue lines indicate the probability of target attainment in plasma, lung, cellular lesions and caseous lesions, respectively. The minimum bactericidal concentration required to achieve 99% kill (MBC₉₉) (5) and the concentration required to inhibit >99% growth in THP1 macrophages (38) are indicated by arrows.
Table 1. Intracellular uptake of EMB in resting and activated primary macrophages of mouse and human origin, and in THP-1 cells

<table>
<thead>
<tr>
<th></th>
<th>Human BMDM</th>
<th>Mouse BMDM</th>
<th>THP-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor 1</td>
<td>Donor 2</td>
<td>Donor 3</td>
</tr>
<tr>
<td>uninfected</td>
<td>21.96 ± 2.78</td>
<td>14.98 ± 2.03</td>
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<td>iMtb</td>
<td>19.64 ± 3.84</td>
<td>11.06 ± 1.55</td>
<td>14.02 ± 9.82</td>
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BMDM: bone marrow derived macrophages; iMtb: infected with γ-irradiated (inactivated) M. tuberculosis; M0: resting macrophages; M1: subjected to classical activation; subjected to alternate activation (see methods)

Table 2. Final model PK parameter estimates for EMB in New Zealand White rabbits receiving 100 mg/kg daily dose. Cl = drug clearance; Vc = central compartment volume; Kcp, Kpc, Kcl, Kccell and Kccas = inter-compartment rate constants where c is central compartment, l is lung, cel is cellular lesion, and cas is caseous or necrotic lesion; Rcl and Rccas = compartmental penetration coefficients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Error (CV%)</th>
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<tbody>
<tr>
<td>Cl (L h⁻¹)</td>
<td>32.4</td>
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<tr>
<td>Vc (L)</td>
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<td>5.60</td>
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<tr>
<td>Kcp (h⁻¹)</td>
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<tr>
<td>Kcc (h⁻¹)</td>
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<tr>
<td>Kcl (h⁻¹)</td>
<td>8.10</td>
<td>9.72</td>
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<tr>
<td>Kccell (h⁻¹)</td>
<td>7.52</td>
<td>25.0</td>
</tr>
<tr>
<td>Kccas (h⁻¹)</td>
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<td>17.6</td>
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<tr>
<td>Rcl</td>
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<tr>
<td>Rccell</td>
<td>3.33</td>
<td>19.9</td>
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Table 3. PK parameters of EMB in 1,000 simulated adult female New Zealand White rabbits receiving 200 mg/kg daily

<table>
<thead>
<tr>
<th>Parameter</th>
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</table>

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<th>Compartment</th>
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<th>AUC₀-2₄ (mg • h mL⁻¹)</th>
<th>T₁/₂ (h)</th>
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<tr>
<td></td>
<td>Estimate</td>
<td>Error (CV%)</td>
<td>Estimate</td>
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<tr>
<td>Plasma</td>
<td>5.32</td>
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<td>Lung</td>
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<td>Cellular lesion</td>
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<tr>
<td>Caseous lesion</td>
<td>17.1</td>
<td>14.8</td>
<td>212</td>
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</table>
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